12-27-99

A

Attorney's Docket No. **102-302 RE/CON** **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of

Inventor(s): **TCHAO, RUY,** a citizen of the U.S.A, whose address is:
404 Cedar Lane, Flourtown, PA 19031

For (title): **CHEMOTAXIS ASSAY PROCEDURE**

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this New Application Transmittal and documents referred to as enclosed herein are being deposited with the United States Postal Service on this date, **December 23, 1999**, in an envelope as "Express Mail to Addressee" Mailing Label Number **EJ094853005US**, addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

M J Mullin
Name of person mailing paper

M J Mullin
Signature of person mailing paper

1. **Type of Application**

This new application is a(n):

[ ] Original (nonprovisional) application.

[ ] Design application.

[ ] Plant application.

[ ] Divisional of Serial No. _____, filed on _____, under

[ ] 37 CFR 1.53

[X] Continuation of copending Serial No. **09/159,427** , filed on **September 23, 1998**, which is a Reissue of **United States Patent No. 5,601,997**, issued **February 11, 1997**, under

[X] 37 CFR 1.53(b)

[ ] Continuation-in-part of Serial No. _____, filed on _____, under

[ ] 37 CFR 1.53

2. **Benefit of Prior U.S. Application(s) (35 U.S.C. 119(e), 120, or 121)**

[ ] This new application claims the benefit of prior U.S. application(s).

[X] Please amend the specification by inserting, before the first line, the following:

[ ] "This application claims the benefit of U.S. Provisional Application No. _____ , filed on _____."

[X] "This application is a
[X] continuation
[ ] continuation-in-part
[ ] divisional
of copending application
[X] Serial No. **09/159,427** , filed on **September 23, 1998**, which is a Reissue of **United States Patent No. 5,601,997**, issued **February 11, 1997**."
[ ] International Application No. _____, filed on _____, and which designated the U.S."

[ ] A Preliminary Amendment is enclosed amending this application to state the relation of this application to prior applications.

[ ] Processing and retention fee ($130.00 (37 CFR 1.53(d) and 1.21(l))) $_____

[ ] Fee for international-type search report ($40.00 (37 CFR 1.21(e))) $_____

Total fees enclosed **$ 1,378.00** .

17. Method of Payment of Fees

[X] Check in the amount of $ **1,378.00** .

[ ] Charge Deposit Account No. in the amount of $_____.
A duplicate of this transmittal is enclosed.

18. Authorization to Charge Additional Fees

[X] The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Deposit Account No. 08-2461:

[X] 37 CFR 1.16(a), (f), or (g) (filing fees)

[X] 37 CFR 1.16(i), (j), and (d) (presentation of extra claims)

[ ] 37 CFR 1.16(e) (surcharge for filing the basic fee and/or declaration at a date later than the filing date of the application)

[ ] 37 CFR 1.17 (application processing fees)

A duplicate of this transmittal is enclosed.

19. Instructions as to Overpayment

[X] Credit Deposit Account 08-2461.

[ ] Refund.

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[X] Reissue Declaration

[X] Copy of Petition for 3 Month Extension of Time filed in Serial No. **09/159,427**, filed on **September 23, 1998**, which is a Reissue of **United States Patent No. 5,601,997**, issued **February 11, 1997**.

[ ] Associate Power of Attorney

[ ] Citations

[ ] Declaration of Biological Deposit

[ ] Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence

[ ] Authorization of Attorney(s) to Accept and Follow Instructions from Representative

[X] Reissue Application by the Inventor, Offer to Surrender (Copy)

[X] Request For Transfer of Drawings From Original Patent to Reissue Application (Copy)

6. Declaration or Oath

[ ] A Declaration or Oath is enclosed, executed by (check all applicable boxes):

[ ] inventor(s).

[ ] legal representative(s) of inventors(s) (37 CFR 1.42 or 1.43).

[ ] joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

[ ] This transmittal serves as the petition required under 37 CFR 1.47, and the statement required under 37 CFR 1.47 is also enclosed. See item 13 below for fee.

[X] A Declaration or Oath was filed on **September 23, 1998** in prior application **09/159,427**, from which benefit is being claimed for this application under 35 U.S.C. 120 or 121. The subject matter disclosed in the present application is the same as that disclosed in the prior application, and the inventors are the same or less than those named in the prior application. Accordingly, no new Oath or Declaration is required.

[X] A copy of the Oath or Declaration in the prior application is enclosed.

[ ] The relation of this application to prior applications is stated in the application.

3. 35 U.S.C. 119 Priority Claim for Prior Application

This application, and prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 2, claim(s) priority from one or more foreign applications as follows:

(Country)	(Application No.)	(Filing Date)
(Country)	(Application No.)	(Filing Date)
(Country)	(Application No.)	(Filing Date)

Certified copy(ies) of the application(s) from which priority is claimed:

[ ] has(have) been filed on _____, in prior application _____, which was filed on _____.
[ ] is (are) enclosed.
[ ] will follow.

4. Papers Enclosed Which are Required to Obtain Application Filing Date under 37 CFR 1.53(b) (Regular) or 37 CFR 1.153 (Design)

10 Pages of specification
6 Pages of claims
1 Pages of Abstract
7 Sheets of drawings
[X] formal
[ ] informal

[ ] The enclosed drawing(s) include photograph(s), and there is also attached a "Petition to Accept Photograph(s) as Drawings." 37 CFR 1.84(b).

5. Additional Papers Enclosed

[ ] Preliminary Amendment

[X] Information Disclosure Statement (37 CFR 1.98)

[X] Form PTO-1449

[X] U.S. Patent No. 5,601,997

[ ] A Declaration or Oath is not enclosed.

[ ] Application is made by a person authorized under 37 CFR 1.41(c) on behalf of *all* of the above named inventor(s).

[X] A Power of Attorney is included in the Declaration or Oath.

7. Language

This new application is written in:

[X] English.

[ ] A non-English language: _____.

[ ] A verified translation is enclosed (37 CFR 1.52(d)).

8. Assignment

[ ] Assignment of the invention

[ ] is enclosed. A separate:

[ ] "Cover Sheet for Assignment (Document) Accompanying New Patent Application" is enclosed.

[ ] Form PTO-1595 is enclosed.

[ ] was made in prior application No._____, filed on _____.

[ ] A copy of the assignment and recordation cover sheet are enclosed.

[ ] will follow.

9. Maintenance of Copendency of Prior Application

[X] A Petition for a 3 month Extension of Time and the appropriate fee has been filed and extends the term in the pending prior application until **December 25, 1999**.

[X] A copy of the petition filed in the prior application is attached.

[ ] A conditional petition for extension of time is being filed in the pending prior application.

[ ] A copy of the conditional petition in the prior application is attached.

10. Abandonment of Prior Application

[X] Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

11. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

[ ] There is provided herewith a Petition to Suspend Prosecution for the Time Necessary to File an Amendment.

12. Amendments

13. Fee Calculation (37 CFR 1.16)

A. [X] Regular application (37 CFR 1.16(a)) Basic Fee $ **760.00**

FEES FOR CLAIMS AS FILED

Number filed		Number extra		Rate		
Total Claims (37 CFR 1.16(j))	**37** - 20 =	**17**	X	$ 18.00	=	$ **306.00**
Independent Claims (37 CFR 1.16(i))	**6** - 2 =	**4**	X	$ 78.00	=	$ **312.00**
Multiple Dependent Claims (37 CFR 1.16(d))		**0**	+	$260.00	=	$ **0**

Fee Calculation for Extra Claims $ **618.00**

[ ] Amendment canceling extra claims enclosed.

[ ] Amendment deleting multiple-dependencies enclosed.

B. [ ] Design application (37 CFR 1.16(f)) Filing Fee $330.00

C. [ ] Plant application (37 CFR 1.16(g)) Filing Fee $540.00

Total Filing Fee Calculation **$ 1378.00**

14. Request for International-Type Search (37 CFR 1.104(d))

[ ] Please prepare an international-type search report for this application at the time national examination on the merits takes place. See item 15 for fee.

15. Small Entity Statement(s)

[ ] A Verified Statement that this is a filing by a small entity under 37 CFR 1.9 and 1.27;

[ ] is enclosed.
[ ] will follow.

[ ] Status as a small entity was claimed in prior application_____, filed on _____, from which benefit is being claimed for this application under:

[ ] 35 U.S.C. 119(e),
[ ] 35 U.S.C. 120,
[ ] 35 U.S.C. 121,
[ ] 35 U.S.C. 365(c),

and which status as a small entity is still proper and desired.

[ ] A copy of the verified statement in the prior application is enclosed.

Filing Fee Calculation (50% of **A**, **B**, or **C** above) $_____

16. Fee Payment Being Made at This Time

[ ] Not enclosed. No filing fee is to be paid at this time.

[X] Enclosed:

[X] Basic filing fee (Item 13 or 15 above) $ **1,378.00**

[ ] Fee for recording Assignment ($40.00 (37 CFR 1.21(h))) $_____

[ ] Petition fee for filing by other than all of the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached. ($130.00 (37 CFR 1.47 and 1.17(h))) $_____

[ ] Fee for processing an application having a specification in a non-English language. ($130.00 (37 CFR 1.52(d) and 1.17(k))) $_____

CHEMOTAXIS ASSAY PROCEDURE

FIELD OF THE INVENTION

This invention relates to a chemotaxis assay procedure and, more particularly, relates to an in vitro chemotaxis assay procedure which is non-destructive of the cell sample and permits kinetic study of the chemotactic response. This invention also relates to a novel radiation opaque membrane for use in the chemotaxis procedure.

BACKGROUND OF THE INVENTION

Chemotaxis is broadly defined as the orientation or movement of an organism or cell in relation to a chemical agent. Chemotaxis assays, particularly in vitro chemotaxis assays, are widely used procedures in medical, biological, pharmaceutical and toxicological research. Such assays are perhaps most widely used to determine the effect of a chemical agent on the inflammatory process, either as a stimulant or inhibitor of that process.

The currently used chemotaxis assay procedure derives from that originally developed by S. Boyden in 1962. (See, S. Boyden, *The Chemotactic Effect of Mixtures of Antibody and Antigen on Polymorphonuclear Leucocytes*, J. Exp. Med. 115: pp. 453–466, 1962). Essentially, the procedure involves placing a suspension of neutrophils and a chemical agent in two separate chambers, which chambers are separated by a polycarbonate filter. The neutrophils are typically either human polymorphonuclear neutrophils ("PMN's") prepared from the peripheral blood of volunteers or PMN's prepared from the peritoneal fluid of animals, such as guinea pigs or rabbits.

After a predetermined period of time, the filter is removed and cells on the filter surface closest to the chamber containing the cell suspension are carefully removed. The remaining cells on the filter are then fixed and stained. Using a high power microscope, the filter is examined and the number of cells appearing on the underside of the filter (i.e., the side of the filter closest to the chamber containing the chemical agent) are counted manually. A positive chemotactic response is indicated by the cells having migrated or "crawled" through the filter to the side closest to the chamber containing the chemical agent. Because of the time required to do so, typically the entire filter is not examined. Rather, representative sample areas are examined and counted.

There are several disadvantages to this procedure. The examination and counting of the cells on the filter is time-consuming, tedious and expensive. It is also highly subjective because it necessarily involves the exercise of judgment in determining whether to count a cell that has only partially migrated across the filter. In addition, the time and expense associated with examining the entire filter necessitates that only representative areas, selected at random, be counted, thus rendering the results less accurate than would otherwise be the case if the entire filter were examined and counted.

Perhaps the most important disadvantage in this procedure is that the fixing step kills the cells. That is, the procedure is destructive of the cell sample. Thus, in order to determine a time-dependent relationship of the chemotactic

response; that is, a kinetic study, of a particular chemical agent, it is necessary to run multiple samples for each of multiple time periods. When one considers that multiple samples, as well as positive and negative controls, are necessary to obtain reliable data, a single chemotaxis assay can produce dozens of filters, each of which needs to be individually examined and counted. The time and expense associated with a time-dependent study is usually prohibitive of conducting such a study using the Boyden procedure.

Alternatives to the Boyden assay have been proposed to overcome some of the above disadvantages. See generally, P. Wilkinson, *Micropore Filter Methods for Leukocyte Chemotaxis*, Methods in Enzymology, Vol. 162, (Academic Press, Inc. 1988), pp. 38–50. See also, Goodwin, U.S. Pat. No. 5,302,515; Guiruis et al., U.S. Pat. No. 4,912,057; Goodwin, U.S. Pat. No. 5,284,753; and Goodwin, U.S. Pat. No. 5,210,021. Although the chemotaxis devices and procedures described in these references have some advantages over the original Boyden procedure and apparatus, they are not without their shortcomings. For example, all of these procedures, like Boyden, require that the filter be removed and the non-migrated cells wiped or brushed from the filter before the migrated cells can be counted. In addition, most of these procedures require fixing and staining the cells and none of them permit the kinetic or time-dependent study of the chemotactic response of the same cell sample.

SUMMARY OF THE INVENTION

I have developed a chemotaxis assay procedure which avoids the above disadvantages, which is non-destructive, and which readily permits kinetic study of the chemotactic response. The chemotaxis procedure of this invention is simple, quick and inexpensive to perform, produces objective data, and is usable with a variety of different cell types.

Basically, the non-destructive chemotaxis assay procedure comprises the steps of:

a) labeling cells with a dye;

b) placing the labeled cells in a first chamber;

c) placing a chemical agent in a second chamber adjacent to said first chamber;

d) separating said first chamber from said second chamber with a radiation opaque membrane, said radiation opaque membrane having a plurality of substantially perpendicular transverse pores therein;

e) stimulating the labeled cells on the side of the membrane closest to said second chamber with electromagnetic radiation of a first wavelength whereby said labeled cells will emit electromagnetic radiation of a second wavelength; and

f) measuring the emitted electromagnetic radiation from the side of the radiation opaque membrane closest to the second chamber; wherein said radiation opaque membrane comprises a film which is not substantially transmissive to at least one of said first and second wavelengths of electromagnetic radiation.

In another aspect, the invention comprises a radiation opaque membrane for use in a chemotaxis assay procedure wherein cells labeled with a dye are stimulated with electromagnetic radiation of a first wavelength whereby the cells

will emit electromagnetic radiation of a second wavelength, said radiation opaque membrane comprising a film which is not substantially transmissive to at least one of said first or second wavelengths of electromagnetic radiation, said radiation opaque membrane having a plurality of substantially perpendicular transverse pores therein.

These and other aspects of the invention will become apparent upon a reading of the following detailed description of the embodiments, with reference to the drawings, and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a perspective view of a preferred apparatus used in carrying out the present procedure.

FIG. 2 is an enlarged, sectioned view of the apparatus of FIG. 1 as seen along line 2—2 of FIG. 1.

FIG. 3A is a simplified schematic view, in cross-section, of cells migrating across one embodiment of the radiation opaque membrane of the present invention.

FIG. 3B is a simplified schematic view, in cross-section, of cells migrating across another embodiment of the radiation opaque membrane of the present invention.

FIGS. 4–7 are graphs of fluorescence units vs. incubation time of the chemotaxis data generated by the Examples.

DETAILED DESCRIPTION OF THE EMBODIMENTS

Although not critical to the present invention, a description of the preferred apparatus for use in carrying out the chemotaxis procedure of this invention is included because it is believed to be helpful in illustrating the advantages of this invention over the prior art. It is to be expressly understood, however, that any number of devices may be used in carrying out the present procedure and the invention is not limited to the use of any particular apparatus, except as set forth in the appended claims.

With reference first being made to FIGS. 1 and 2, the preferred apparatus comprises a multi-well culture plate which is widely available from a variety of commercial sources. This type of apparatus is commonly employed to study the effects of chemical agents on cell growth. As seen in FIGS. 1 and 2, the apparatus comprises a plate 20 having a plurality of spaced-apart wells 22. Each well 22 is provided with an insert 24 adapted to fit inside the well. In the parlance of this specification, the interior of the insert comprises one chamber and the exterior of the insert comprises a second chamber. The size, shape and number of wells 22, inserts 24, and plate 20 are not critical to this invention.

For purposes of this invention, the bottom of the insert 24 has been provided with a radiation opaque membrane 10 of this invention, which separates the two chambers. The radiation opaque membrane 10 may be attached to the bottom of the insert by any conventional means, such as glue or other adhesive, heat welding, ultrasonic welding, etc. In practice, the labeled cells are placed in the insert 24 and the chemical agent is placed in the well 22. The chemotactic reaction will cause the labeled cells to migrate or "crawl" from the chamber 24 to chamber 22, through the pores 16 in

the radiation opaque membrane 10, as particularly shown in FIGS. 3A and 3B.

As seen in FIG. 2, a space 28 is created between the radiation opaque membrane 10 and the bottom of the well 22. A distance of about 1 mm between the bottom of well 22 and the radiation opaque membrane 10 is generally sufficient to permit the free migration of cells across the radiation opaque membrane. The space 28 may be conveniently created by providing the insert 24 with stand-offs 26, which may take any convenient form or shape (e.g. legs, bosses, flange, etc.). When using stand-offs, care should be taken not to isolate the fluid in space 28 from the remainder of the fluid in the well 22, which would tend to create a separate concentration gradient in the space 28. Alternatively, the space 28 may be created by suspending the insert 24 within the well 22 by the use of, for example, radial projections 27 which rest on the surface of plate 20 as shown in FIGS. 1 and 2.

At predetermined periods, the quantum of cells that have migrated across the radiation opaque membrane will be determined by first exciting or stimulating the labeled cells on the side of the radiation opaque membrane 10 closest to the chamber 22 and measuring the radiation emitted by those labeled cells. With the preferred apparatus illustrated in FIGS. 1 and 2, this step would comprise stimulating and measuring the radiation from below the radiation opaque membrane 10, that is, through space 28. It will be understood by those skilled in the art that it is preferred that at least the chamber through which the stimulation and measurement of radiation will take place is substantially transparent to both the radiation being measured and any radiation needed to excite or stimulate the dye used to label the cells. In the preferred embodiment, the apparatus is made of a clear, transparent material, such as polystyrene, polycarbonate, LUCITE®, glass, etc.

The device 30 used to stimulate the cells and measure the emitted radiation will, of course, depend on the dye used to label the cells and the type of apparatus used for the assay procedure. For example, if the plate apparatus of FIGS. 1 and 2 is used, a fluorescent plate reader, such as a Cytofluor™ 2300 (Millipore Corp., Marlborough, MA), can be used to advantage. The radiation opaque membrane 10 will substantially prevent either the stimulation of the cells in chamber 24 or the transmission of radiation from the cell sample in chamber 24 into the space 28, or will prevent both. Accordingly, the radiation measured will provide a direct quantitative measure of the number of cells that have migrated across the radiation opaque membrane 10 from chamber 24 to chamber 22.

It will be appreciated by those skilled in the art that neither insert 24, nor radiation opaque membrane 10, nor the non-migrated cells adhered to it, need be removed prior to measuring the radiation corresponding to the migrated cells. This permits repeated measurements of the chemotactic response of the same cell sample, thus permitting simple and rapid quantitative determinations in a kinetic, or time-dependent, profile of the chemotactic response with a minimum number of test samples. In addition, the devices used to measure the radiation, such as plate readers or spectrophotometers, are highly sensitive and accurate pieces of equipment and provide objective data corresponding to the

number of migrated cells. This is a distinct advantage over the prior art procedures which rely upon subjective physical examination under a microscope.

As mentioned above, the chemotaxis assay of this invention can be used with a variety of cell types. Examples include, but are not limited to, macrophages, eosinophils, fibroblasts, endothelial cells, epithelial cells, PMN's, tumor cells and prokaryotic organisms. The only practical limitations on the cell type are its ability to exhibit a chemotactic response and its ability to be labeled.

In accordance with the present invention, the cell sample is labeled with a fluorescent dye. The process of labeling cells with dyes is well known, as is the variety of fluorescent dyes that may be used for labeling particular cell types. See e.g. R. Haugland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Inc. (1989). A particularly preferred fluorescent dye for use with an HL-60 cell line (ATCC No. CCL 240) in the present invention is Di-I (Molecular Probes, Inc.; Eugene, OR).

It should be mentioned here that, in theory, non-fluorescent dyes may be used in the present invention. At the present time however, there are no known devices that can be used to measure the transmitted light from migrated cells to the exclusion of the transmitted light from the non-migrated cells. Accordingly, the practical utility of using non-fluorescent dyes in the present invention awaits the discovery or invention of such a device.

A particularly novel aspect of the present invention is the use of a radiation opaque membrane which is not substantially transmissive to at least the wavelength of electromagnetic radiation used to stimulate the labeled cells or the wavelength of electromagnetic radiation emitted by the labeled cells. Preferably, the radiation opaque membrane is not substantially transmissive to both wavelengths of electromagnetic radiation, which would protect against excitation of non-migrated cells and would also prevent transmission of radiation emitted by any non-migrated cells that may, nevertheless, become stimulated. It may be advantageous in certain situations, such as for example where mixed cell types and multiple labeling dyes are used, to selectively block either the excitation wavelength or the emission Wavelength. Because the radiation opaque membrane is porous, it will be impossible to completely block all transmission of radiation across the radiation opaque membrane, simply because some radiation will be transmitted through the pores in the radiation opaque membrane. In practice, however, the quantum of radiation so transmitted will be relatively constant and negligible in terms of the quantum of radiation radiating from the migrated cells. Generally speaking, however, the radiation opaque membrane (absent any pores) should have a blocking efficiency of at least approximately 95%. That is, the membrane should be capable of blocking at least approximately 95% of the intended radiation, either the radiation used to stimulate the cells, the radiation emitted by the labeled cells, or the combined stimulation and emission radiation.

In accordance with the present invention, such membranes permit the measurement of radiation emitted from the labeled cells that have migrated through the radiation opaque membrane without interference from radiation emitted from the labeled cells that have not migrated, without the

need to remove the non-migrated cells from the radiation opaque membrane. This is a significant advantage of the present invention over the prior art procedures, not only because it avoids the tedious steps of removing the filter and scraping the non-migrated cells from the filter, but also because it is non-destructive of the cell sample and thus permits repeated measurements of the same test sample at different time intervals.

The radiation opaque membrane itself may be of any convenient construction, so long as it has the properties mentioned above. In general, the radiation opaque membrane **10** comprises a non-fibrous film **12** of polyester, polycarbonate, polyethylene terephthalate, polylactic acid, nylon, etc. Depending on the type of film used, the film may be dyed to obtain the radiation blocking properties discussed above. In lieu of or in addition to using a dyed film, one or more radiation blocking layers **14** may be applied to the film by any conventional process suitable for the particular film and blocking layer(s) being used, such as coating under vacuum, layer transfer, sputtering, spin coating, vacuum deposition, etc. The thickness of the radiation opaque membrane **10** is not critical to the invention. Membranes having a thickness in the range customarily used in the art are suitable for use herein.

As already noted, the radiation opaque membrane must have a plurality of pores **16** disposed substantially perpendicular to the plane of the radiation opaque membrane to permit the migration of cells across the radiation opaque membrane. The diameter of the pores is not particularly critical and, to a large extent, depends upon the size of the cells being studied. Generally speaking, the pores **16** must be of such diameter to prevent the cells from passively traversing the radiation opaque membrane while at the same permitting the cells to actively "crawl" through the radiation opaque membrane. It is readily within the skill of the ordinary artisan to determine the appropriate pore size for a particular chemotaxis assay without undue experimentation. Pores of suitable size can be provided in the film by any known process, such as atomic etching. If a radiation blocking layer(s) is to be applied to the film, it may be done either before or after the pores have been provided.

EXAMPLES

Cell Sample

The cell line HL-60 (ATCC No. CCL 240) was maintained in logarithmic growth phase as a suspension culture at about 10^6 cells/mL in RPMI 1640 medium (Mediatech Cellgrow, Fisher Scientific, Pittsburgh, PA.) supplemented with 20% (volume by volume) fetal bovine serum. (Hyclone Laboratories, Salt Lake City, UT). The cells were differentiated into mature myelocytes and neutrophils by incubating the cells for 48 hours at 37° C. in media containing 1.5% (volume by volume) dimethylsulfoxide.

Cell Labeling

Following the treatment with dimethylsulfoxide, the cells were incubated with 50 uM Di-I fluorescent dye (Molecular Probes, Inc., Eugene, OR) at room temperature for 0.5–2 hours. The cells were then washed with Hanks' Balanced

Salt Solution ("HBSS") (Sigma Chemical Co., St. Louis, MO.) and re-suspended in HBSS to achieve a cell concentration of 10^6 cells/mL. The fluorescence of 0.5 mL. of cell suspension was measured in a Cytofluor™ 2300 fluorescent plate reader (Millipore Corp., Marlborough, MA.).

Membrane Preparation

Membrane 1: Polycarbonate film having a plurality of pores of 8 μm diameter were coated with four molecular layers of carbon and one layer of an admixture of gold and palladium in a vacuum evaporator. The resulting radiation opaque membrane had a thickness of about 10 μm and was approximately 97% efficient in blocking the combined stimulation and emission radiation. 6 mm disks of the radiation opaque membrane were glued to the bottom of inserts similar to the Millicell HA-12 mm (Millipore Corp.) or the Transwell-6.5 mm (Costar Corp., Cambridge, MA.) inserts with clear silicone rubber cement.

Membrane 2: A non-porous polyester film(18 μm thick) containing a blue dye (Aquired Technology Inc., Alpharetta, GA.) was subjected to atomic etching to produce a 10 μm thick radiation opaque membrane containing a plurality of pores of 8 μm diameter having a combined radiation blocking efficiency of approximately 99%. 6 mm disks of the radiation opaque membrane were glued to the bottoms of inserts as with membrane 1.

Test Procedure

Each insert equipped with the either membrane 1 or membrane 2 were placed in a well of a 24-well culture plate (Falcon, Fisher Scientific). 0.5 mL of labeled cell suspension was placed inside each insert. The plate was incubated for 30 minutes at 37° C. to allow the cells to settle on the radiation opaque membrane. The fluorescence of each well was then measured with the Cytofluor™ 2300 to obtain a zero time reading. 0.5 mL of either N-formyl methionyl leucyl phenylalanine ("f-MLP") (Sigma Chemical Co.) or HBBS was

then added to each well. The fluorescence in each well was then measured at periodic time intervals using the Cytofluor™ 2300 at sensitivity setting 4. Results using membrane 1 are reported in Tables 1 and 2 and graphically illustrated in FIGS. 4 and 5. Results using membrane 2 are reported in Table 3 and graphically illustrated in FIGS. 6 and 7.

TABLE 1

Well Number	Test Solutions well/insert	Fluorescence 0 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
1	HBSS/HBSS	546	757	862	922	927	904
2	HBSS/f-MLP[1]	383	1046	1355	1433	1370	1359
3	f-MLP[2]/f-MLP	706	654	708	732	728	753
4	f-MLP[2]/f-MLP	467	412	435	460	447	454
5	Blank	130	124	125	125	125	125
6	Blank	132	127	127	128	127	126
7	Blank	131	127	127	128	127	126
8	Blank	128	124	126	125	127	125
9	Blank	129	125	126	126	126	125
10	Blank	130	127	127	127	128	127
11	Blank	135	133	132	132	132	132
12	Blank	130	126	125	126	125	125
13	Blank	132	128	129	130	129	128
14	Blank	134	141	136	139	136	137
15	Blank	137	134	133	134	134	132
16	Blank	136	131	132	133	132	132
17	Blank	135	134	132	134	131	132
18	Blank	137	132	131	132	132	133
19	Blank	136	132	131	132	132	133
20	Blank	139	135	132	135	134	135
21	Blank	141	135	136	138	136	137
22	Blank	140	137	137	138	136	137
23	0.5 mL cells	9999[3]	9999	9999	9999	9999	9999
24	0.5 mL cells	9999	9999	9999	9999	9999	9999

Notes:
1. Conc. = 10^{-7} M
2 f-MLP added to cell suspension immediately before start of experiment.
3. Fluorescence was greater than measurable at selected sensitivity setting.

TABLE 2

Well Number	Test Solutions insert/well	FLUORESCENCE 0 min.	15 min.	30 min.	60 min.	90 min.	120 min.	150 min.
1	HBSS/f-MLP[1]	2927	4195	4475	4642	4761	4801	4788
2	HBSS/f-MLP	2895	4165	4400	4539	4642	4681	4655
3	HBSS/f-MLP	2631	3398	3584	3645	3728	3759	3728
4	HBSS/f-MLP	2594	3446	3707	3813	3932	3988	3999
5	HBSS/f-MLP	2515	3388	3594	3614	3717	3759	3770
6	f-MLP[2]/F-MLP	2854	2675	2721	2721	2783	2783	2783
7	HBSS/HBSS	2558	2683	2736	2783	2862	2886	2911
8	HBSS/HBSS	2862	2977	3028	3053	3114	3132	3105
9	HBSS/HBSS	3105	3194	3221	3220	3294	3313	3294
10	HBSS/HBSS	2377	2660	2767	2846	2927	2952	2960
11	Blank	165	163	162	163	160	160	160
12	Blank	166	163	164	162	161	160	657
13	Blank	166	163	163	163	158	161	157
14	Blank	166	166	163	163	163	162	159
15	Blank	162	160	160	159	156	157	156
16	Blank	163	160	159	159	156	157	153
17	Blank	162	161	160	159	158	156	156
18	Blank	164	161	162	159	158	151	147
19	Blank	163	161	162	158	158	158	154
20	Blank	162	161	160	159	158	153	145
21	Blank	168	166	165	163	163	163	160
22	Blank	165	164	163	159	160	151	151

TABLE 2-continued

Well Number	Test Solutions insert/well	FLUORESCENCE 0 min.	15 min.	30 min.	60 min.	90 min.	120 min.	150 min.
23	Blank	171	168	168	164	150	166	162
24	Blank	172	170	169	153	151	162	162

Notes:
1. Conc. = 10^{-8} M
2. f-MLP added to cell suspension immediately before start of experiment

TABLE 3

Well Number	Test Solutions insert/well	Fluorescence 0 hr.	0.5 hr.	1 hr.	2 hr.	3 hr.
1	HBSS/HBSS[1]	1079	1378	1586	1770	1810
2	HBSS/HBSS	891	1058	1194	1351	1421
3	HBSS/HBSS	940	1221	1382	1533	1617
4	Blank	178	169	169	167	166
5	f-MLP[2]/f-MLP	961	1245	1390	1564	1711
6	0.3 mL cells	9999	9999	9999	9999	9999
7	HBSS/f-MLP[3]	1055	1770	2066	2351	2536
8	HBSS/f-MLP	1064	1454	1846	2143	2292
9	HBSS/f-MLP	1097	1775	2185	2411	2432
10	Blank	178	187	196	191	190
11	f-MLP/f-MLP	1049	1277	1413	1538	1582
12	0.3 mL cells	9999[4]	9999	9999	9999	9999
13	HBSS/HBSS[5]	1425	1491	1577	1682	1735
14	HBSS/HBSS	1359	1454	1491	1551	1645
15	HBSS/HBSS	1340	1386	1478	1582	1650
16	Blank	179	172	176	172	171
17	f-MLP/f-MLP	1187	1181	1516	1622	1673
18	0.4 mL cells	9999	9999	9999	9999	9999
19	HBSS/f-MLP	1277	1573	1701	1836	1851
20	HBSS/f-MLP[6]	1228	5928	6063	6342	6504
21	HBSS/f-MLP	1242	1207	1830	1931	1969
22	Blank	176	171	169	167	166
23	f-MLP/f-MLP	1231	1325	1454	1541	1604
24	0.4 mL cells	9999	9999	9999	9999	9999

Notes:
1. Transwell-type inserts used for wells 1–12.
2. f-MLP added to cell suspension immediately before start of experiment.
3. Conc. = 2×10^{-8} M
4. Fluorescence greater than measurable at selected sensitivity setting.
5. Millicell-type inserts used for wells 13–24.
6. Insert leaked

What is claimed is:

1. A non-destructive chemotaxis assay procedure comprising the steps of:

a) labeling cells with a fluorescent dye;

b) placing the labeled cells in a first chamber;

c) placing a chemical agent in a second chamber adjacent to said first chamber, said chemical agent being capable of inducing migration of said labeled cells from said first chamber to said second chamber;

d) separating said first chamber from said second chamber with a radiation opaque membrane, said radiation opaque membrane having a plurality of substantially perpendicular transverse pores therein;

e) stimulating the labeled cells on the side of the membrane closest to said second chamber with electromag-

netic radiation of a first wavelength whereby said labeled cells will emit electromagnetic radiation of a second wavelength; and

f) measuring the emitted electromagnetic radiation from the side of the radiation opaque membrane closest to the second chamber; wherein said radiation opaque membrane comprises a film which is not substantially transmissive to at least one of said first and second wavelengths of electromagnetic radiation.

2. The procedure of claim 1, wherein the fluorescent dye is Di-I.

3. The procedure of claim 3, wherein the radiation opaque membrane comprises a polyester film containing a blue dye.

4. The procedure of claim 1, wherein the radiation opaque membrane comprises a polycarbonate film coated with four layers of carbon and one layer of an admixture of gold and palladium.

5. The procedure of claim 1, wherein step (f) comprises measuring the electromagnetic radiation with a fluorescent plate reader.

6. The procedure of claim 1, further comprising the step of repeating steps (e) and (f) at least once at a predetermined time interval.

7. The procedure of claim 6, wherein the dye comprises a fluorescent dye.

8. The procedure of claim 7, wherein the fluorescent dye is Di-I.

9. The procedure of claim 8, wherein the radiation opaque membrane comprises a polyester film containing a blue dye.

10. The procedure of claim 8, wherein the radiation opaque membrane comprises a polycarbonate film coated with four layers of molecular carbon and one layer of an admixture of gold and palladium.

11. The procedure of claim 7, wherein the step (f) comprises measuring the electromagnetic radiation with a fluorescent plate reader.

12. The procedure of claim 6, wherein the film has a radiation blocking efficiency of at least approximately 95%.

13. The procedure of claim 1, wherein the film has a radiation blocking efficiency of at least approximately 95%.

14. The procedure of claim 13, wherein the film has a radiation blocking efficiency of at least approximately 97%.

15. A chemotaxis assay procedure comprising measuring the migration of cells across a radiation opaque membrane, wherein said procedure is non-destructive of said cells.

* * * * *

[57] ABSTRACT

A chemotaxis assay procedure which is non-destructive of the cells being studied, which permits the ready performance of kinetic or time-dependent study of cell migration from the same sample, and which produces objective measurements includes the steps of:(a)labeling cells with a dye; (b) placing the labeled cells in a first chamber; (c) placing a chemical agent in a second chamber adjacent to said first chamber; (d) separating said first chamber from said second chamber with a radiation opaque membrane, said radiation opaque membrane having a plurality of substantially perpendicular transverse pores therein; (e) stimulating the labeled cells on the side of the membrane closest to said second chamber with electromagnetic radiation of a first wavelength whereby said labeled cells will emit electromagnetic radiation of a second wavelength; and (f) measuring the emitted electromagnetic radiation from the side of the radiation opaque membrane closest to the second chamber; wherein said radiation opaque membrane comprises a film which is not substantially transmissive to at least one of said first and second wavelengths of electromagnetic radiation. The radiation opaque membrane may comprise a dyed film or a film which has at least one radiation blocking layer applied thereto.

15 Claims, 7 Drawing Sheets

WHAT IS CLAIMED IS:

16. An assay that is non-destructive of a sample being detected comprising the steps of:

labeling said sample;

placing said labeled sample in a first chamber;

separating said first chamber from said second chamber with a permeable, detection-blocking membrane;

measuring sample presence in said second chamber by detecting said labeled sample in said second chamber without substantially detecting said labeled sample in said first chamber.

17. An assay of claim 16 further including the step of:

inducing migration of said labeled sample across said membrane.

18. An assay of claim 17 wherein said inducing step includes:

placing a chemical agent in said second chamber capable of creating a chemotactic reaction with said sample.

19. An assay of claim 18 wherein said sample includes cells.

20. An assay of claim 18 wherein said labeling step includes:

labeling said cells with a dye.

21. An assay of claim 20 wherein said measuring step includes:

measuring radiation emitted by said labeled cells in said second chamber without substantially measuring radiation emitted by said labeled cells in said first chamber.

22. An assay of claim 21 wherein said dye is a fluorinated dye and wherein said inducing step includes:

stimulating said labeled cells in said second chamber with electromagnetic radiation of a first wavelength whereby said labeled cells emit electromagnetic radiation of a second wavelength; and

measuring said electromagnetic radiation of said second wavelength from said cells in said second chamber wherein said detection-blocking membrane is a radiation opaque member which is not substantially transmissive to at least one of said first and second wavelength of electromagnetic radiation.

23. A non-destructive sample assay comprising:
measuring sample migration across a permeable detection-blocking membrane.

24. A non-destructive sample assay of claim 23 further including the step of:
inducing migration of said sample across said membrane.

25. A non-destructive sample assay of claim 24 wherein said inducing step includes:
providing a chemotactic agent on one side of said membrane.

26. A non-destructive sample assay of claim 24 wherein said measuring step includes:
detecting sample presence on said one side of said membrane.

27. A non-destructive assay comprising:

detecting sample presence on one side of a sample-porous detection-blocking barrier without substantially detecting sample presence on the other side of said barrier.

28. A non-destructive assay of claim 27 further including:

inducing migration of said sample through said sample-porous detection-blocking barrier.

29. A non-destructive assay of claim 28 wherein said sample migration is induced by a chemotactic agent.

30. An assay for detecting the presence of a material which has traversed a porous membrane comprising measuring or detecting the presence of said material which has migrated across said porous membrane wherein said membrane substantially prevents the detection and/or measurement of any said material which has not migrated across said membrane, and wherein said assay is non-destructive of said material.

31. A non-destructive assay for detecting the presence of a labeled sample comprising:

a) incorporating identification means into said biological sample;

b) placing said biological sample in a first chamber, said first chamber being separated from a second chamber by a porous radiation opaque membrane that comprises a film which is not substantially transmissive to a detection source used to detect said identification means;

c) allowing said biological sample to migrate across said membrane in response to a signal;

d) detecting sub populations of said biological sample in said second chamber but not said first chamber based on the presence of said identification means.

32. The non-destructive assay of claim 31, wherein said sample is selected from the group consisting of primary and tissue culture cells.

33. The non-destructive assay of claim 32, wherein said identification means is selected from the group consisting of dyes, radio-active labels and magnetic labels.

34. The non-destructive assay of claim 31, wherein said sub populations are selected from the group consisting of cells, cellular products, bacteria, viruses, and viral particles.

35. The non-destructive assay of claim 34, wherein said cellular products include proteins, protein fragments, nucleic acids, and nucleic acid fragments.

36. The non-destructive assay of claim 31, wherein said signal is selected from the group consisting of chemical signals, electrical signals, electromagnetic signals and magnetic signals.

37. An assay for identifying and/or quantitating the presence of a labeled analyte from a sample comprising measuring the presence of said labeled analyte in a second chamber, wherein said second chamber is isolated from a first chamber by a porous radiation opaque membrane, wherein said membrane comprises a film which is not substantially transmissive to a detection means used to detect said labeled analyte and said assay is non-destructive of said analyte.

COPY

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application for Reissue of U.S. Patent No. 5,601,997

Applicant(s): Tchao, R.	Examiner: Wong, L. (Anticipated)
Serial No.:	Group Art Unit: 1302 (Anticipated)
Filed:	Docket: 102-302 Re
For: Chemotaxis Assay	Dated: September 23, 1998

Assistant Commissioner for Patents
Washington, DC 20231

REISSUE DECLARATION

Sir:

I, Ruy Tchao, declare that I am a citizen of the United States and a resident of Flourtown, Pennsylvania, and:

that I believe that I am the original and first sole inventor of the subject matter claimed in U.S. Patent No. 5,601,997 (hereinafter the '997 patent), entitled "Chemotaxis Assay Procedure";

that I have reviewed and understand the specification of the accompanying reissue application, including the claims;

that I believe that I am the original and first sole inventor of the subject matter which is claimed and for which a reissue patent is sought; and

that I acknowledge my duty to disclose to the U.S. Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56.

I further declare that I believe the above-identified original patent to be partly inoperative or invalid by reason of my claiming less than I had a right to claim in the original patent.

Specifically, I believe that the original patent is partly inoperative or invalid for including limitations in the claims which were not required by the prior art.

During the course of negotiations to license certain of my rights in the '997 patent to Becton Dickinson and Company (hereinafter "Becton") certain limitations in the claims thereof came to my attention. In particular, after reviewing the specification and issued claims of U.S. Patent No. 5,601,997 and consulting with patent attorneys at Becton and the law firm of Hoffmann & Baron, LLP, who are outside patent counsel for Becton, I came to the realization that I had inadvertently failed to claim certain broad aspects of my invention.

I believe that the error constituted inadvertent failure to appreciate the full scope of claims which were available in view of the prior art, and that the error arose without any deceptive intention on my part.

Claim 16 of the reissue application corresponds generally with claim 1 of the '997 patent. Claim 16, however, differs from claim 1 in at least one respect including, for example:

The limitation "relating to the use of a radiation opaque membrane" included in claim 1 does not appear in claim 16. Claim 16 additionally recites the use of a "permeable, detection blocking membrane". No art was cited against this claim 1 by the Examiner.

These and other limitations in claim 1 resulted from the apparent failure of myself and prior patent counsel to fully appreciate the limiting nature of the claim limitations, as well as failure to fully appreciate the full scope of the invention as taught by the specification.

In the course of attempting to license my patent to Becton, I was advised by Becton's outside counsel of the narrow scope of coverage to which the '997 patent may be entitled as a result of such limitations. During prosecution of the application, I did not fully realize or

appreciate the effect of these limitations on the scope of coverage provided by the resulting patent.

I have always felt that a significant aspect of my invention is its applicability to any assay which lends itself to the use of a permeable, detection blocking membrane for detecting and/or identifying and/or quantifying biological material present on one side of the membrane but without detecting/identifying/quantifying biological material that has not crossed the membrane. Furthermore, I have always believed that another significant aspect of my invention was to provide an assay that was substantially non-destructive of the biological material that was being detected/identified/quantified notwithstanding the nature of the assay. Through inadvertent error, none of the claims of the '997 patent either as originally submitted or as allowed, claim these specific aspects of my invention.

Furthermore, all errors being corrected in the present reissue application arose without any deceptive intention on my part.

I declare that all statements made herein of my own knowledge are true, and that all statements made upon information and belief are believed to be true, and further that these statements were made after being warned that wilful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such false statements may jeopardize the validity of this application or any patent issuing thereon.

Full name of sole or first inventor Ruy Tchao, Ph.D.

Inventor's Signature Ruy Tchao Date Sept. 21 1998

Residence 404 Cedar Lane, Flourtown, PA 19031 Citizenship U.S.A.

Post Office Address _____

Practitioner's Docket No. 102-302 **PATENT**

REQUEST FOR TRANSFER OF DRAWINGS FROM ORIGINAL PATENT TO REISSUE APPLICATION

Please transfer the drawings from original patent, 5,601,997, filed on February 3, 1995, for the invention entitled Chemotaxis Assay Procedure

to the reissue application, the specification of which:

- ☒ is attached hereto.
- ☐ was filed on _____, as reissue application number /

Signature of practitioner

Date: September 22, 1998

Kevin C. Hooper
(type or print name of practitioner)

Reg. No.: 40,402

HOFFMANN & BARON, LLP
350 Jericho Turnpike
Jericho, NY 11753
P.O. Address

Tel. No. (973) 331-1700

Customer No.:

COPY

Practitioner's Docket No. 102-302 Re **PATENT**

REISSUE APPLICATION BY THE INVENTOR, OFFER TO SURRENDER (37 C.F.R. § 1.178)

To the Assistant Commissioner for Patents:

1: The undersigned applicant of the accompanying reissue application for the reissue of letters patent for the improvement in Chemotaxis Assay Procedure
Patent number 5,601,997 granted to him/her on February 11, 1997, of which

☒ he/she is now sole owner,

☐ _____

is now sole owner by assignment, and on whose behalf and with whose assent the accompanying application is made,

☐ The "ASSENT BY THE ASSIGNEE" to this reissue application is attached.

Date: 9/21/98

Ruy Tchao
Signature(s)

Ruy Tchao
(type or print name(s))

CERTIFICATION UNDER 37 C.F.R. § 1.10*
(Express Mail label number is mandatory.)
(Express Mail certification is optional.)

I hereby certify that this correspondence and the documents reffered to as attached therein are being deposited with the United States Postal Service on this date 9/23/98 in an envelope as "Express Mail Post Office to Addressee," service under 37 C.F.R. § 1.10, Mailing Label Number EI855061016US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

JENNIFER BURKS
(type or print name of person mailing paper)

Jennifer Burks
Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

***WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

Practitioner's Docket No. 102-302 Re **PATENT**

REISSUE APPLICATION DECLARATION AND POWER OF ATTORNEY (BY INVENTOR(S) OR ASSIGNEE)

(complete A or B)

COPY

A. ☒ DECLARATION BY THE INVENTOR(S)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor *(if only one name is listed below)* or an original, first and joint inventor *(if plural names are listed below)* of the subject matter that is described and claimed in letters patent number 5,601,997, granted on February 11, 1997, and for which invention I solicit a reissue patent on the invention entitled Chemotaxis Assay Procedure

the specification of which

☒ is attached hereto.

☐ was filed on _____, as reissue application number / and was amended on _____ *(if applicable).*

☒ I hereby declare that there is no assignee for this application.

NOTE: "Where no assignee exists, applicant should affirmatively state that fact. If the file record is silent as to the existence of an assignee, it will be presumed that no assignee exists." M.P.E.P., 6th ed., rev. 1, § 1410.01.

B. ☐ DECLARATION BY ASSIGNEE

NOTE: The assignee of the entire interest may make the declaration, if the reissue application does not seek to enlarge the scope of the claims of the original patent. 37 C.F.R. § 1.172.

_____, _____

(type or print name of declarant) Title

of _____,

Name of company or legal entity on whose behalf declarant is authorized to sign

declare that I am a citizen of _____ and resident of _____,

_____, that the entire title to letters patent number _____,

for _____,

granted on _____, 19____ to _____

Inventor(s)

is vested in _____

Name of company or legal entity

that I believe said named inventor(s) to be an original, first and sole inventor *(if only one name is listed)* or an original, first and part inventor *(if plural names are listed)* of the subject matter that is described and claimed in the aforesaid letters patent and in the foregoing specification and for which invention I solicit a reissue patent.

ACKNOWLEDGEMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR
(37 C.F.R. § 1.175)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

☒ In compliance with this duty, there is attached an information disclosure statement in accordance with 37 C.F.R. § 1.98.

PRIORITY CLAIM

NOTE: A "claim" for the benefit of an earlier filing date in a foreign country under 35 U.S.C. 119(a)-(d) must be made in a reissue application even though such a claim was made in the application on which the original was granted. However, no additional certified copy of the foreign application is necessary. M.P.E.P., 6th ed., rev. 1, § 1417.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

(complete C or D)

C. ☒ No such applications have been filed.

D. ☐ Such applications have been filed as follows:

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION

Country	Application No.	Date of filing (day, month, year)	Date of issue (day, month, year)	Priority Claimed
				☐ YES NO ☐
				☐ YES NO ☐
				☐ YES NO ☐

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION

BENEFIT OF PROVISIONAL APPLICATION

POWER OF ATTORNEY

I hereby appoint the following practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Charles R. Hoffmann, Reg. No. 24,102; Ronald J. Baron, Reg. No. 29,281; Gerald T. Bodner, Reg. No. 30,449; Alan M. Sack, Reg. No. 31,874; A. Thomas Kammer, Reg. No. 28,226; Arlene D. Morris, Reg. No. 32,657; R. Glenn Schroeder, Reg. No. 34,720; Glenn T. Henneberger, Reg. No. 36,074; Livia Boyadjian, Reg. No. 34,781; Jessica H. Tran, Reg. No. 40,842; Irving N. Feit, Reg. No. 28,601; Anthony E. Bennett, Reg. No. 40,910; Gregory A. Bachmann, Reg. No. P41,593; Steven T. Zuschlag, Reg. No. P43,309, Susan A. Sipos, Reg. No. P43,128; William D. Schmidt, Reg. No. 39,492; and Kevin E. McDermott each of them of HOFFMANN & BARON, 350 Jericho Turnpike, Jericho, New York 11753; and Daniel A. Scola, Jr., Reg. No. 29,855; Salvatore J. Abbruzzese, Reg. No. 30,152; Kirk M. Miles, Reg. No. 37,891; Kevin C. Hooper, Reg. No. 40,402; and Robert F. Chisholm, Reg. No. 39,939, each of them of HOFFMANN & BARON, 1055 Parsippany Boulevard, Parsippany, New Jersey 07054.

(check the following item, if applicable)

☐ I hereby appoint the practitioner(s) associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

☐ Attached, as part of this declaration and power of attorney, is the authorization of the above-named practitioner(s) to accept and follow instructions from my representative(s).

SEND CORRESPONDENCE TO

☒ Address
HOFFMANN & BARON, LLP
350 Jericho Turnpike
Jericho, NY 11753

☐ Customer Number _____

DIRECT TELEPHONE CALLS TO:
(Name and telephone number)

Kevin C. Hooper
973-331-1700

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature(s)

☒ **BY THE INVENTOR(S)**

Full name of sole or first inventor Ruy Tchao

Inventor's signature Ruy Tchao

Date Sept. 21, 1998 Country of Citizenship U.S.

Residence 404 Cedar Lane, Flourtown, PA 19031

Post Office Address Same as Above

Full name of second joint inventor, if any _____

Inventor's signature _____

Date _____ Country of Citizenship _____

Residence _____

Post Office Address _____

☐ **BY ASSIGNEE OR PERSON AUTHORIZED TO SIGN ON BEHALF OF ASSIGNEE**

NOTE: Even though inventor(s) do not sign, complete above information for inventor(s).

(complete the following, if applicable)

_____
(type name of assignee)

_____
Address of assignee

_____

_____
Title of person authorized to sign on behalf of assignee

☐ Assignment recorded in PTO on _____

Reel _____

Frame _____

☐ A separate ☐ "ASSIGNMENT (DOCUMENT) COVER SHEET" or ☐ FORM PTO 1595 is submitted herewith along with the assignment _____.

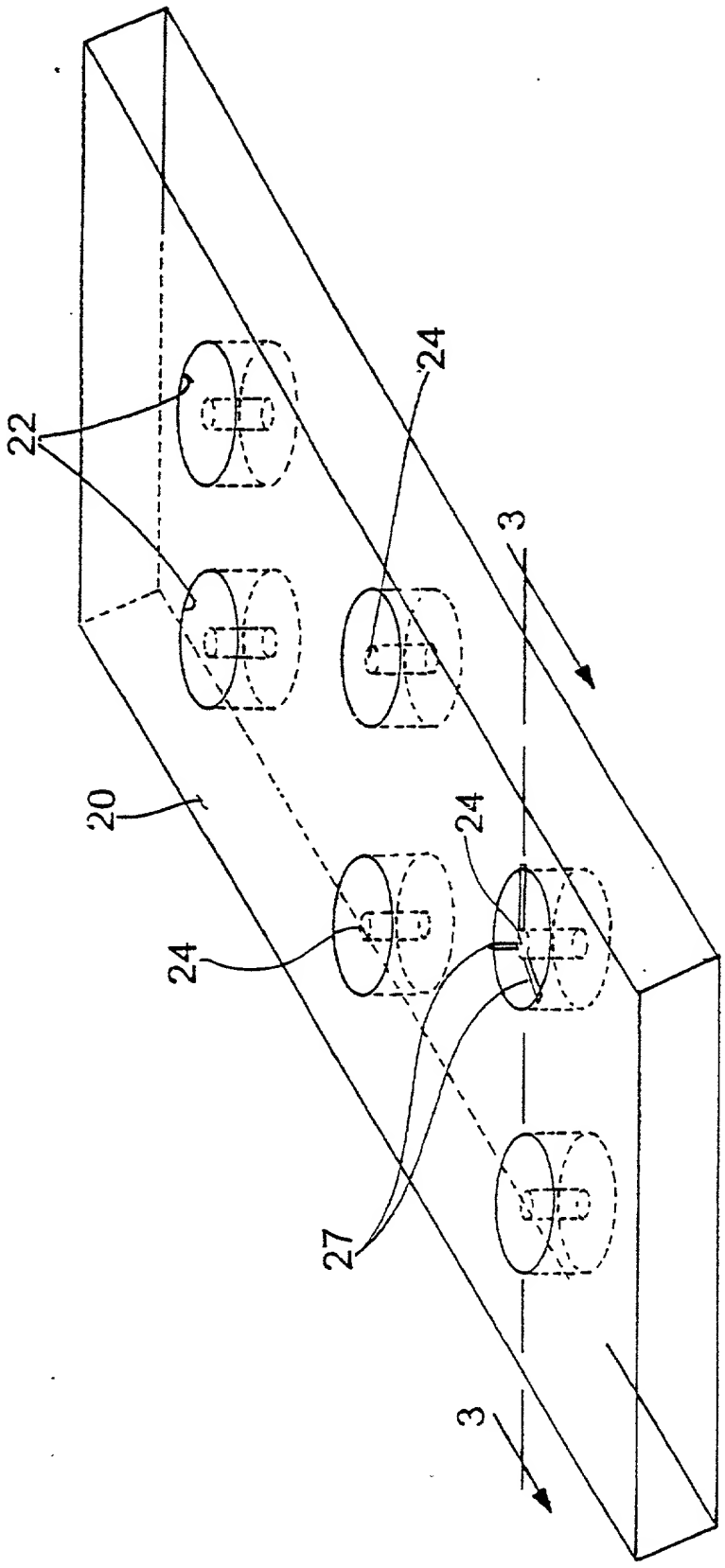


Fig. 1

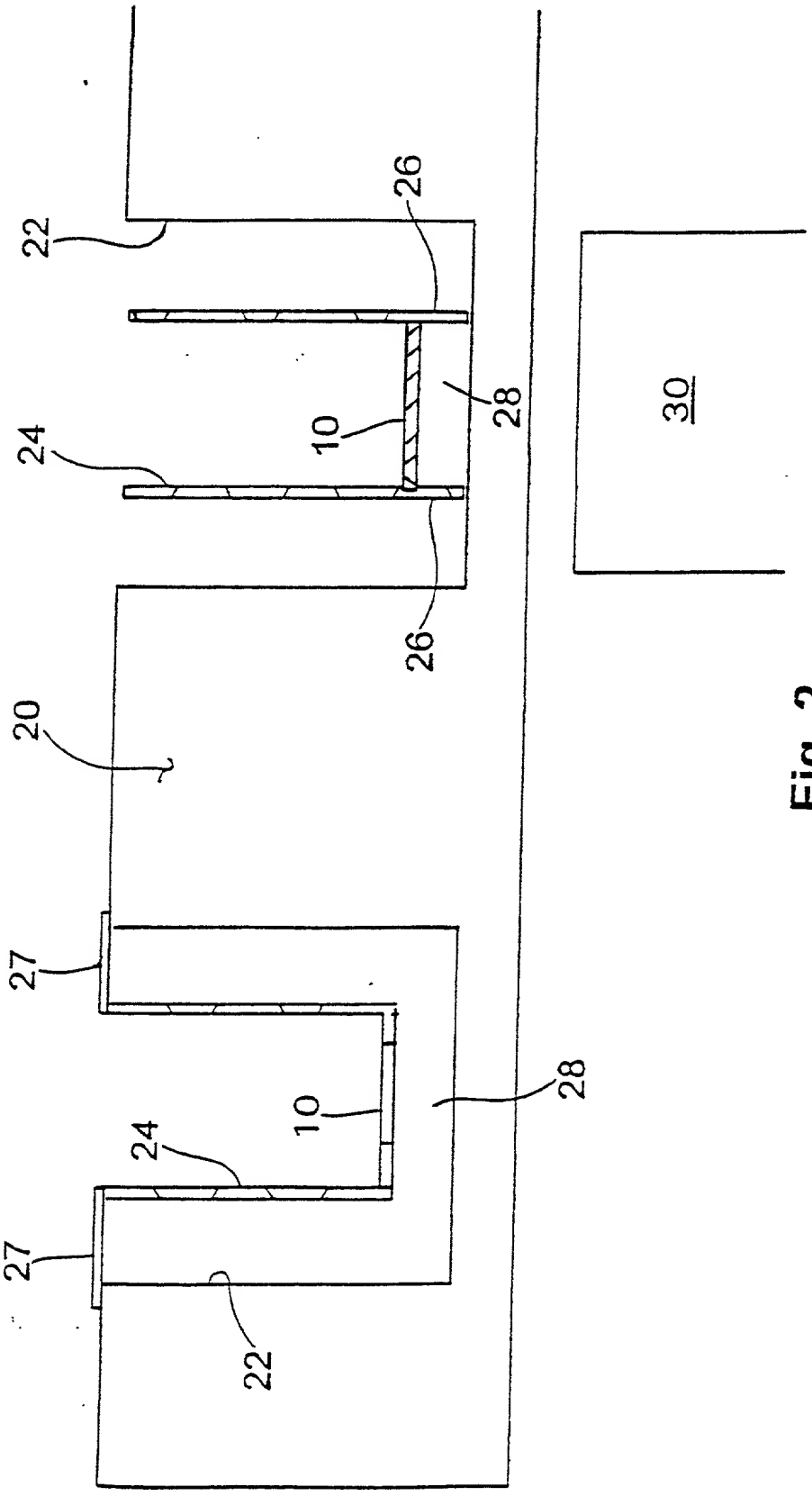


Fig. 2

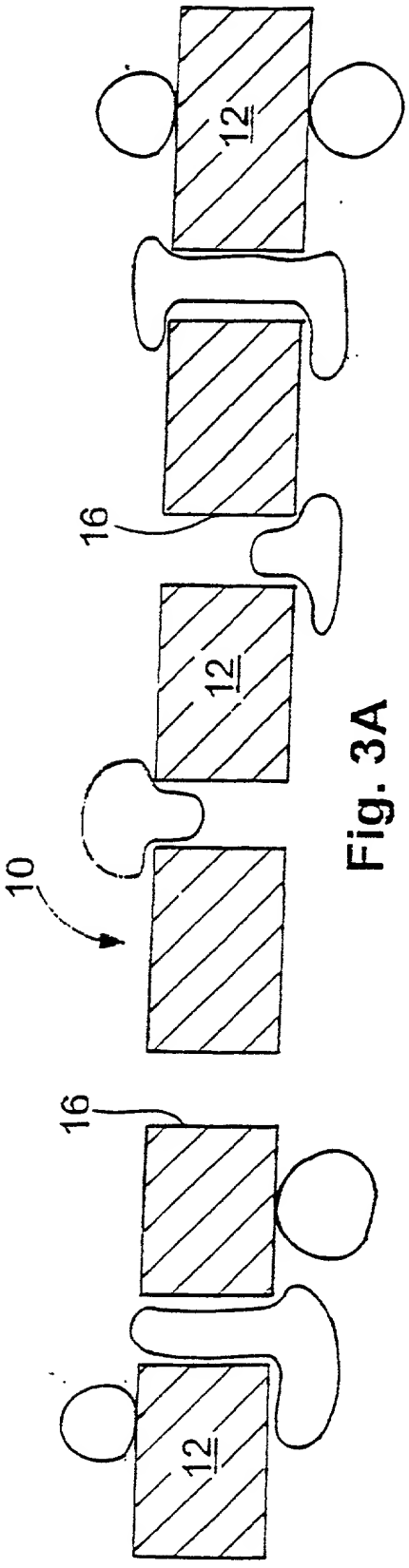


Fig. 3A

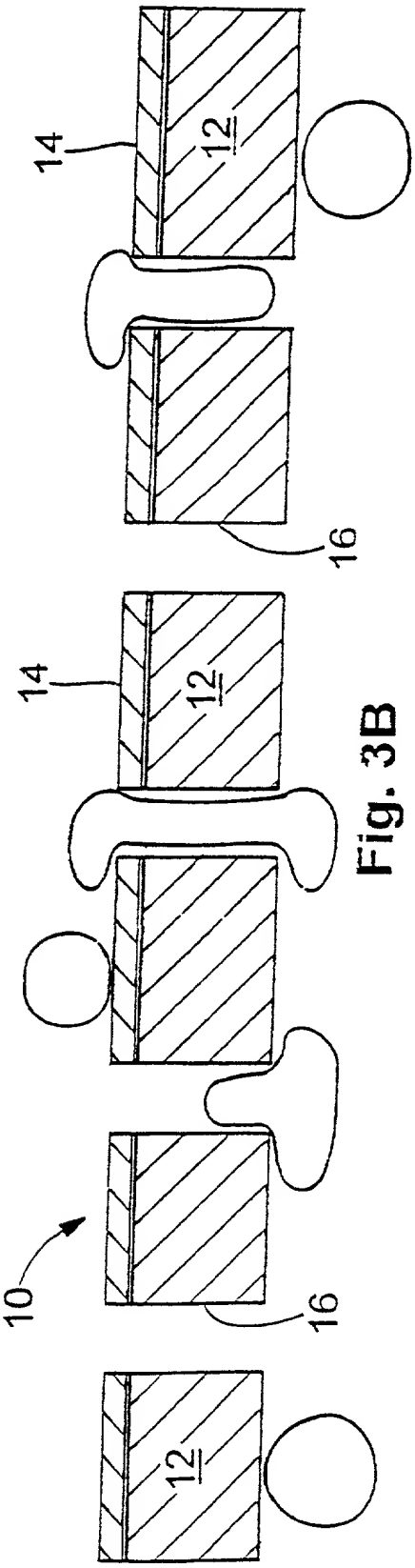


Fig. 3B

Fig. 4

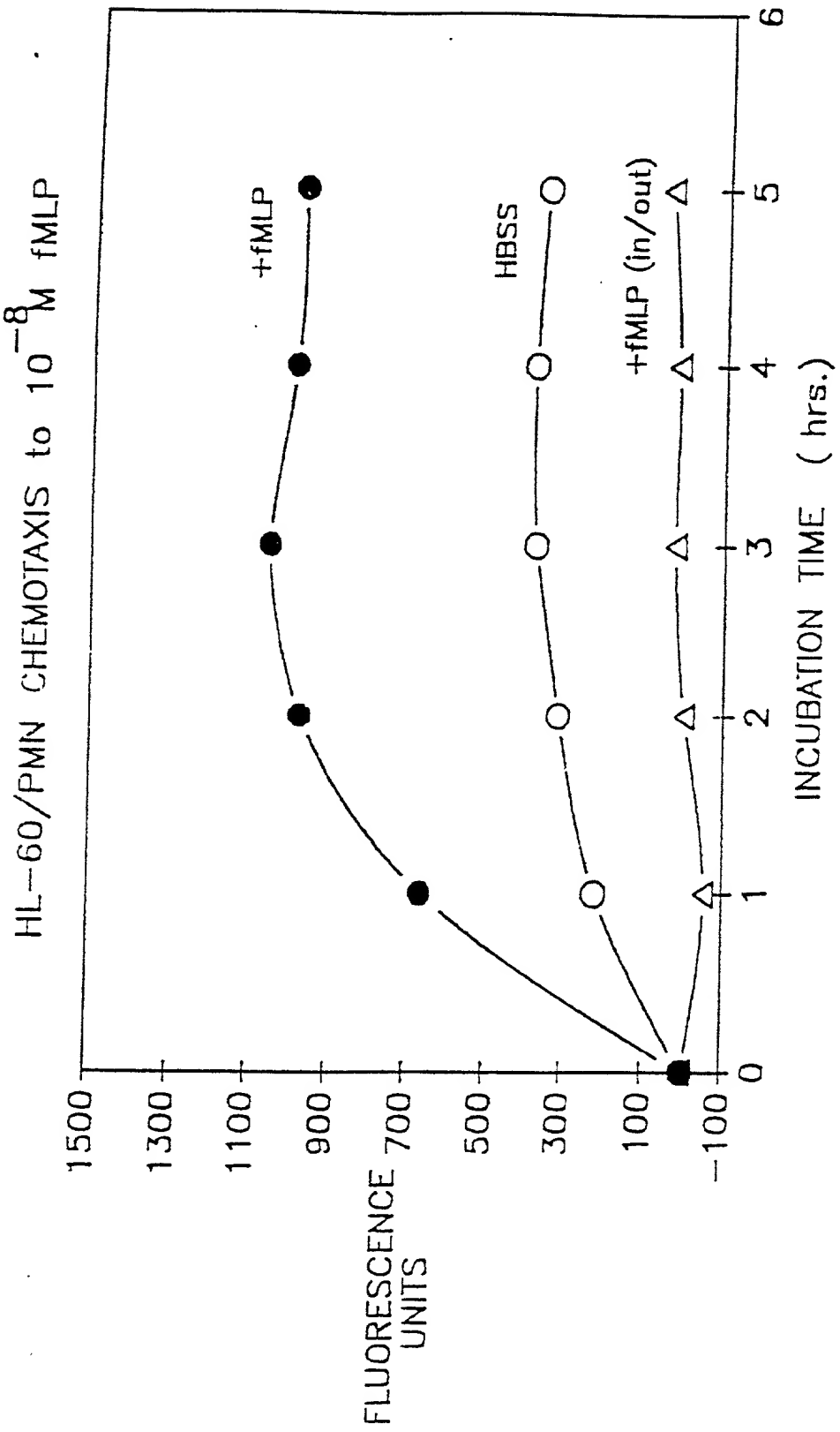

HL-60/PMN CHEMOTAXIS to 10^{-8}M fMLP
+fMLP
HBSS
+fMLP (in/out)
FLUORESCENCE UNITS
1500
1300
1100
900
700
500
300
100
-100
0
1
2
3
4
5
6
INCUBATION TIME (hrs.)

Fig. 5

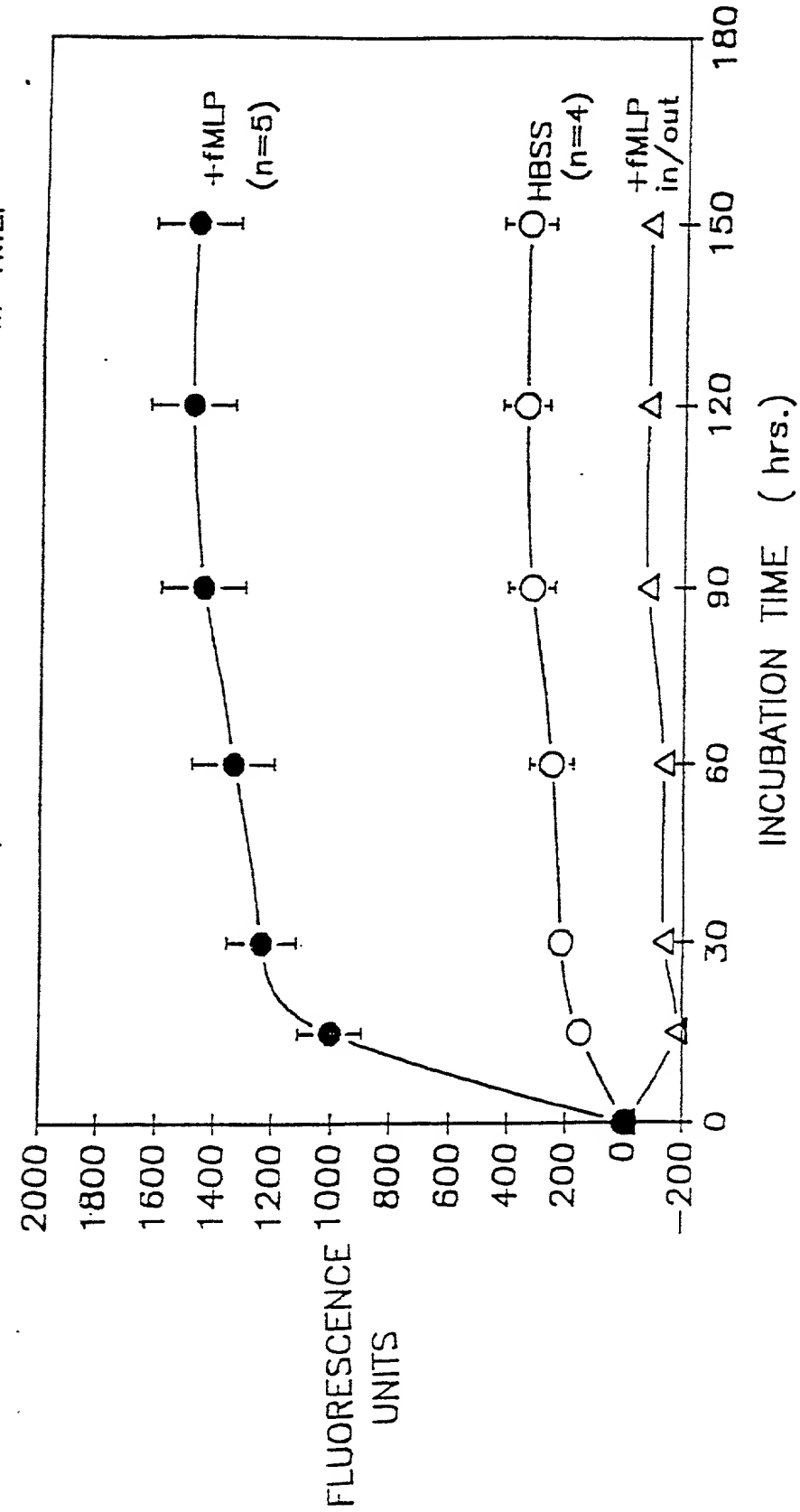

HL-60/PMN CHEMOTAXIS to 10^{-8}M fMLP
+fMLP (n=5)
HBSS (n=4)
+fMLP in/out
FLUORESCENCE UNITS
INCUBATION TIME (hrs.)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
0
30
60
90
120
150
180

Fig. 6

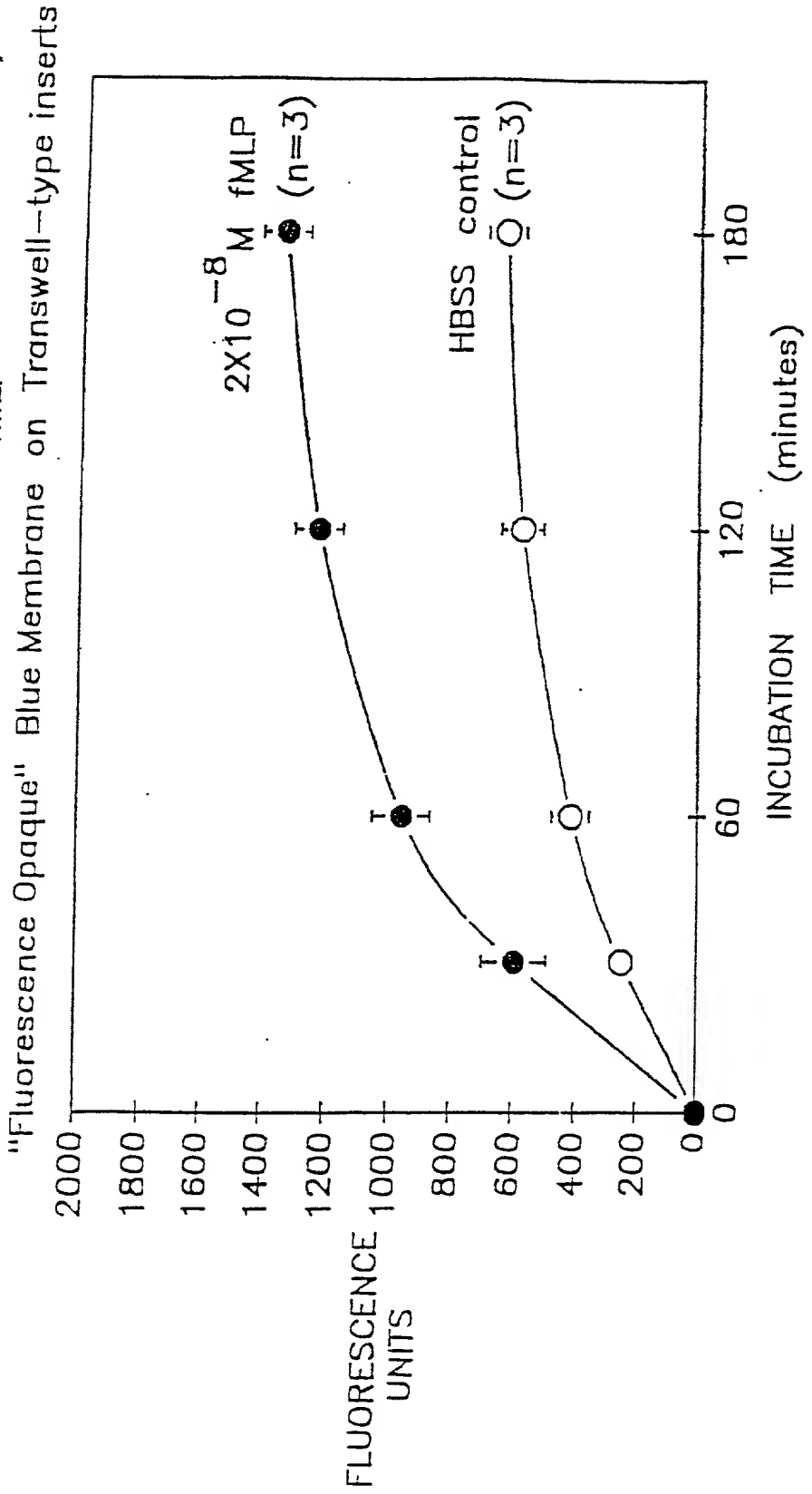
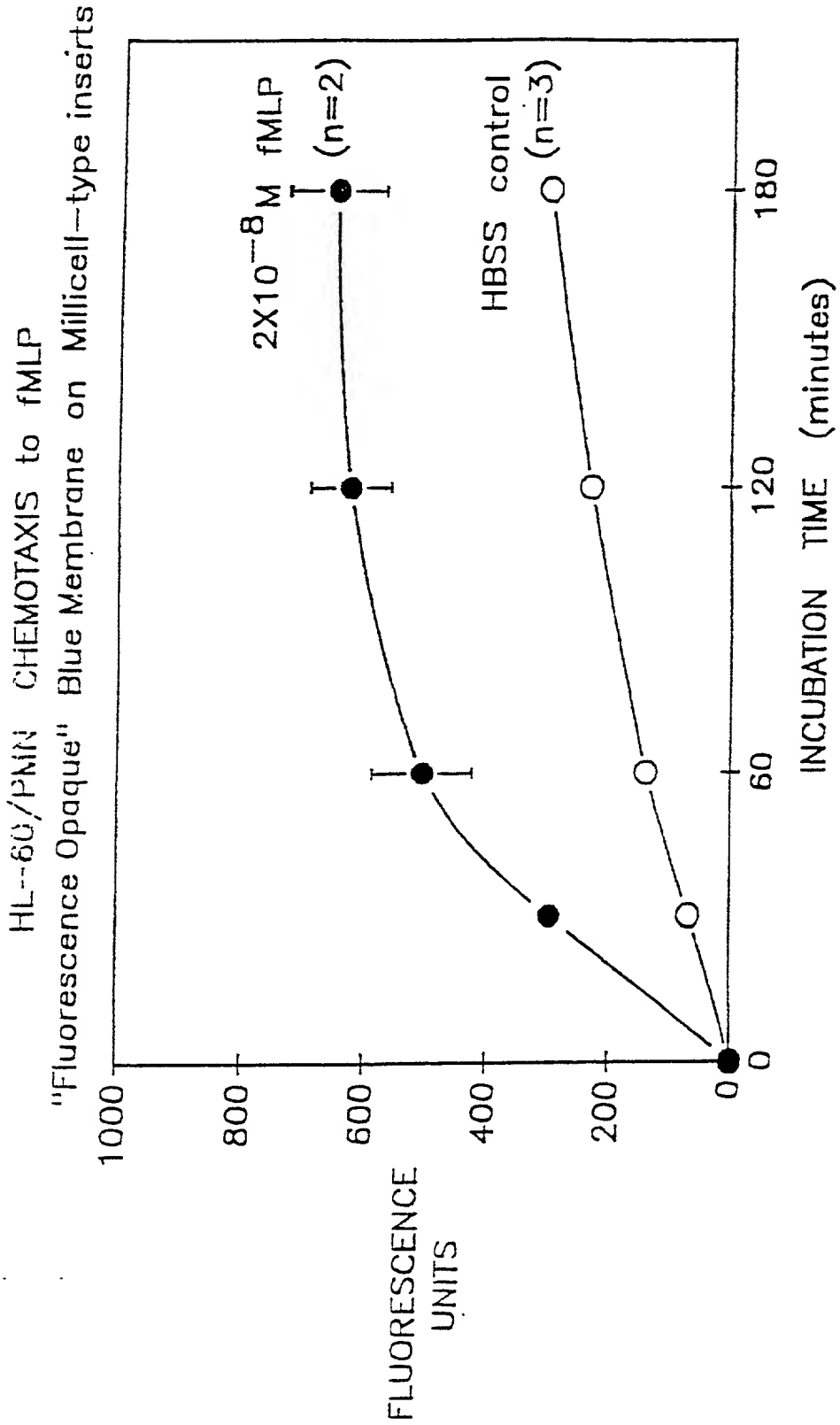

HL-60/PMN CHEMOTAXIS to fMLP
"Fluorescence Opaque" Blue Membrane on Transwell-type inserts
2X10 -8 M fMLP (n=3)
HBSS control (n=3)
FLUORESCENCE UNITS
INCUBATION TIME (minutes)
0
200
400
600
800
1000
1200
1400
1600
1800
2000
60
120
180

Fig. 7


HL-60/PMN CHEMOTAXIS to fMLP
"Fluorescence Opaque" Blue Membrane on Millicell-type inserts
2X10^{-8}M fMLP (n=2)
HBSS control (n=3)
FLUORESCENCE UNITS
1000
800
600
400
200
0
0
60
120
180
INCUBATION TIME (minutes)